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ASSESSMENT OF GENETIC DIVERSITY AMONG INDIAN FENUGREEK (*TRIGOI ELLA FOENUM-GRAECUM* L.) VARIETIES USING MORPHOLOGICAL AND RAPD MARKERS

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ABSTRACT

The study was conducted to reveal genetic variability among the released varieties of fenugreek from different regions of India using morphological and molecular markers. Seventeen varieties were evaluated for important characteristics like plant height, test weight, total weight, yield per plant, pod length, pod per plant, pod per axis, petiole length and 50% flowering. In this study, Random Amplified Polymorphic DNA (RAPD) markers were used to assess genetic diversity wherein fifteen polymorphic primers showed a 57.66% polymorphism. All the varieties were classified into two major clusters viz., cluster-I and cluster-II, cluster-I is further divided into five sub clusters containing twelve varieties namely Hisar Suvarna, Hisar Sonali, Rajendra Kranti, Hisar Mukta, Hisar Madhavi, AM-2, GM-2, AM-1, Azad Methi, CO-2, RMT-143 and RMT-351 showing genetic similarity irrespective of their geographical distribution. Cluster-II contains five varieties viz., Lam Selection, RMT-1, RMT-305, RMT-303 and Pant Ragini. In morphological analysis few varieties did not follow the pattern of RAPD analysis indicating their phenotypic characters to be influenced by the environmental factors. Morphological dendrogram also showed two major clusters and Pant Ragini was found to branch out distinctly confirming its uniqueness from all other varieties.

Key words: Molecular marker, RAPD, Fenugreek, Diversity analysis.

INTRODUCTION

Fenugreek (*Trigonella foenum-graecum* L., 2n= 2x= 16**)** is an important leguminous spice and well known aromatic and medicinal herb. Fenugreek is used as both seed and leaf. It is commonly used to flavor liquors, bread, fish, salad, soups, cheese, curry and manufacturing of pickles, perfumes, soap, cosmetics and cough syrup. It is native to South-eastern Europe and Western Asia and is widely cultivated in India which harbors its great diversity. A large number of germplasm in India are maintained by National Research Centre on Seed Spices (NRCSS), Ajmer and National Bureau of Plant Genetic Resources (NBPGR), New Delhi, India.

Germplasm pool is an important source for developing new cultivars having desirable traits such as higher productivity and quality. The genetic diversity between populations can be identified using morphological, biochemical and molecular markers but morphological and biochemical traits have some limitations as they are highly influenced by environmental factors and the developmental stages of the plants. The PCR based method for DNA profiling, random amplified polymorphic DNA (RAPD) techniques (Welsh & McClelland, 1990; Mir Ali & Nabulus, 2003, Fracaro *et al.*, 2005) have been extensively applied in assessment of genetic diversity of various plant species and is also quite helpful in detecting genetic variability within short time (Khan *et al.*, 2005). RAPD is the very popular and widely used class of molecular marker and uses 10-base primer to amplify the random portion of genome (Williams et al, 1990). RAPD is also most widely exploited by the horticulturists largely due to the fact that results are obtained quickly and are fairly inexpensive to generate (Meerow 2005). Another advantage of RAPD is that it does not require any prior information of target genome. RAPD analysis plays a key role in the study of DNA profile and

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detects the extent of polymorphism within species (Staub *et al.*, 1997). These PCR based markers reveal variation at DNA level, those variations that are obtained from the huge extent of genetic polymorphism generated by these markers, leading to evaluation of phenotypic variability. It is a dominant class of marker useful for cultivar identification.

MATERIALS AND METHODS

Plant Materials: Seventeen most prominent Indian varieties of fenugreek were used in this study (Table 2a). Good quality DNA was isolated from fresh leaves of the fenugreek plants. Approximately 20-25 fresh leaves per plant were collected and genomic DNA was isolated using standard procedure of CTAB method. After checking the quality and quantity, DNA was dissolved in double distilled water to a final concentration of 50ng/ml and stored at -20^{0}C.

PCR Programme and RAPD Analysis: Polymerase chain reaction (PCR) amplification was carried out by MJ PTC 100 thermal cycler (MJ Research Inc., Waltham, MA, USA). The reaction mixture of 25ml contained 50ng genomic DNA, 0.5 unit Taq DNA polymerase, and 1x reaction buffer (containing 1.5 mM of $MgCl_2$). The PCR profile involved initial denaturation at 94°C for 4 min, followed by 32 cycles of 94°C for 1 min, 1.5 min at annealing temperature (depending on RAPDs primers used, Table 1) and 72°C for 2 min. This was followed by a final extension step at 72°C for 10 min.

Gel electrophoresis of the amplified products: Ten micro liters of loading buffer was added into each amplified sample and mixed well. 15 ml of each amplified sample was loaded on 1.5 % agarose gel in 1x TBE buffer to separate the amplified fragments. The electrophoresis was done for 1 and ½ hours at 80 volts. 5 ml of molecular marker (1 bp, 100 bp DNA ladder) was also loaded to compare the molecular weight of amplified products. After electrophoresis, gel was observed and visualized under UV and photographs of gel were taken with the help of Gel- Documentation system.

Data analysis: Total number of band within each line and number of polymorphic bands were noted. Each DNA fragment amplified by a given primer was considered as a unit character and the RAPD fragments were scored as a binary variable (1) for presence and (0) for absence of each of the primer accession combination. Since DNA samples consisted of bulk DNA extracted from individual plants, a low intensity of any particular fragment may be explained by the lesser representation of that specific sequence in the bulk sample of DNATherefore, the intensity of bands was not taken into account and the fragments with the identical mobility were considered but those identical fragments were scored which have only major bands and faint bands were not considered. The accessions were scored for the presence or absence of RAPD bands. The presence or absence of polymorphic and non-polymorphic bands was scored in a binary data matrix. To study the genetic similarity and dissimilarity principal coordinate analysis (PCA) and clustering of RAPD data was done. To check the morphological behavior with the genetic traits clustering based on morphological character using WARD'S method and its cross validation was also done. PCA result was very similar to the cluster obtained by RAPD data using dendrogram. All the statistical analysis were performed using NTSYS 2.20f, SAS 9.2 and JMP Genomics 4 software.

RESULT AND DISCUSSION

A wide variation was recorded for all the 13 morphological traits (Table 2a and 2b). On the basis of leaf size, leaf colour, shape, plant height and plant type all the selected varieties could be distinguished. Based on the pair-wise comparison among the selected varieties, a matrix of Euclidean distance was obtained and a dendrogram was constructed (Fig 1). A perusal of the dendrogram indicates two major clusters, I and II. Cluster-I contains five varieties and Cluster-II contains eleven varieties, Pant Ragini is a dual purpose and tall bushy variety from Uttarakhand branched out from the dendrogram confirming that it is different from all other varieties. Same results were obtained by the RAPD (Fig 2) and PCA analysis (Fig 3). The most similar varieties were CO-2 and Lam Selection falling in the cluster-II both the varieties are for seed purpose. Varieties from different agroclimatic regions are falling into the same cluster and varieties from the same region are falling into the different clusters indicating that the morphological characters are influenced by the environmental conditions. Cluster comparison (Table 3) generated using morphological data also supports these findings.

TABLE 1: Polymorphism shown by OPB series of RAPD primers with primer sequence information

Primer	Sequence (5'-3')	MW	AT (^{0}C)	GC %	Total no of fragments	polymorphic fragment	% of polymorphism
OPB-01	GTTTCGCTCC	2,970.0	33.4	60%	-	-	-
OPB-02	TGATCCCTGG	3,019.0	32.2	60%	9	7	77.77
OPB-03	CATCCCCCT	2,923.9	35.1	70%	13	2	15.38
OPB-04	GGACTGGAGT	3,108.1	32.2	60%	15	10	66.66
OPB-05	TGCGCCCTTC	2,955.0	41.1	70%	5	3	60.00
OPB-06	TGCTCTGCCC	2,955.0	39.8	70%	5	3	60.00
OPB-07	GGTGACGCAG	3,093.1	38.1	70%	14	10	71.42
OPB-08	GTCCACACGG	3,013.0	37.3	70%	-	-	-
OPB-09	TGGGGGACTC	3,084.0	37.0	70%	-	-	-
OPB-10	CTGCTGGGAC	3,044.0	36.6	70%	15	0	0.00
OPB-11	GTAGACCCGT	3,028.0	32.6	60%	8	6	75.00
OPB-12	CCTTGACGCA	2,988.0	35.7	60%	14	9	64.28
OPB-13	TTCCCCCGCT	2,914.9	41.8	70%	14	7	50.00
OPB-14	TCCGCTCTGG	2,995.0	38.8	70%	12	8	66.66
OPB-15	GGAGGGTGTT	3,139.1	33.2	60%	12	9	75.00
OPB-16	TTTGCCCGGA	3,010.0	38.0	60%	6	4	66.66
OPB-17	AAGGAACGAG	3,126.1	33.1	50%	8	6	75.00
OPB-18	CCACAGCAGT	2,997.0	34.3	60%	13	10	76.92
OPB-19	ACCCCCGAAA	2,982.0	38.4	60%	-	-	-
OPB-20	GGACCCTTAC	2,988.0	29.7	60%	-	-	-
		Total			163	94	
		Average			10.86	6.26	57.66

MW: Molecular weight, AT: Annealing Temperature

The analysis of the prescreening data using seventeen fenugreek varieties and 20 RAPD primers showed that 15 primers generated bright and reproducible amplified products, which detected polymorphism among the varieties used. Results of amplification pattern obtained by RAPD in different varieties of fenugreek are shown in Fig 4. In this study, 20 primers having 10 bp of OPB series from 20 operon technologies (OBP-1 to OBP-20) produced polymorphism and all varieties were distinguishable by unique RAPD profiles. These data indicated that the selected RAPD markers were efficient for assessment of genetic diversity in fenugreek.

As fifteen polymorphic primers generated a total of 163 reproducible and scoreable amplicons across the seventeen varieties with the 15 primers (OPB-2, OPB-3, OPB-4, OPB-5, OPB-6, OPB-7, OPB-10, OPB-11, OPB-12, OPB-13, OPB-14, OPB-15, OPB-16, OPB-17 and OPB-18, Table 1). Some of the primers show a high level of genetic diversity while others produced a minor variability. The number of bands per primer ranged from 5 (OPB-5 and 6) -15 (OPB-4 and 10) with an average of 10.86 scoreable bands per primer. Out of the 163 bands, 94 were polymorphic (57.66%) revealing a high degree of polymorphism (Table 1). The number of RAPD bands detected by each primer depends on primers, sequence and extent of variation in specific genotype (Shiran *et al*, 2007) therefore the number of bands varied in different accession. The number of bands generated varied from 5 to 15 with an average of 6.26 bands per primer. The primers OPB-2 gave maximum polymorphism with 77.77% followed by OPB-18 (76.92%), OPB-11 (75%), OPB-15 (75%), OPB-17 (75%), OPB-7 (71.42%), OPB-4 (66.66%), OPB-14 (66.66%), OPB-16 (66.66%), OPB-12 (64.28%), OPB-5 (60%), OPB-6 (60%), OPB-13 (50%) and OPB-3 (15.38%). Primer OPB-10 generated only monomorphic bands. The percentage of polymorphism varied from 15.38% to 77.77% with an average of 57.66%.

TABLE 2A: Qualitative characteristics of different fenugreek varieties under studies.

Variety	State	Parentage	Purpose	Plant Type	Plant Color	Seed Size	Seed Color
Hisar Suvarna	Haryana	Pure line selection from local germplasm by CCS Haryana	Seed	Erect	Green	Medium Bold	Yellow
Hisar Sonali	Haryana	Pure line selection from local germplasm by CCS Haryana	Seed	Semi Erect	Green	Medium Bold	Yellow
AM-2	Rajasthan	Pure line from local germplasm from NRCSS	leaf	Tall Bushy	Yellow	Small	Golden Yellow
RMT-351	Rajasthan	Pure line from local germplasm by SKN college	Dual purpose	Erect	Green	Small	Yellow
Azad Methi	UP	Unknown	Seed	Erect	Purplish Green	Bold	Green
Hisar Mukta	Haryana	IL-335-1 germplasm line	Seed	Erect	Green	Bold	Green
CO-2	Tamil Nadu	TG 2336 germplasm line	Seed	Erect	Green	Medium	Golden
AM-1	Rajasthan	Pure line from local germplasm at NRCSS	Leaf	Semi Erect	Green	Bold	Yellow
Hisar Madhavi	Haryana	PLM E46-1 line	Seed	Erect	Purplish Green	Bold	Green
GM-2	Gujarat	Unknown	Seed	Erect	Green	Medium Small	Yellow
Rajendra Kranti	Bihar	Mass selection from Raghunathpur germplasm	Seed	Bushy	Green	Medium Small	Yellow
RMT-143	Rajasthan	Pure line selection from Jodhpur region	Seed	Erect	Green	Medium Small	Yellow
Lam Selection	Andhra Pradesh	Germplasm collected from MP	Seed	Bushy	Green	Medium	Golden Yellow
RMT-1	Rajasthan	Pure selection from local, Jobner collection	Seed	Semi Erect, Tall	Green	Medium Bold	Yellow
RMT-305	Rajasthan	RMT1	Seed	Bushy, Dwarf	Green	Medium Bold	Yellow
RMT-303	Rajasthan	Unknown	Seed	Erect	Green	Medium Bold	Yellow
Pant Ragini	Uttarakhand	Unknown	Dual purpose	Tall Bushy	Green	Medium Small	Yellow

TABLE 2b: Quantitative characteristics of different fenugreek varieties under studies.

	Quantitative characteristics												
Variety	1	2	3	4	5	6	7	8	9	10	11	12	13
Hisar Suvarna	51.00	0.69	5.00	6.00	13.00	25.00	10.00	16.00	63.00	85.00	596.00	39.00	14.00
Hisar Sonali	50.00	1.00	6.00	9.00	12.00	35.00	11.00	19.00	68.00	102.00	646.00	44.00	15.00
AM-2	48.00	0.91	5.00	9.00	12.00	33.00	10.00	19.00	66.00	102.00	538.00	46.00	15.00
RMT-351	50.00	1.03	5.00	10.00	11.00	35.00	10.00	17.00	68.00	104.00	696.00	66.00	17.00
Azad Methi	50.00	0.96	6.00	10.00	12.00	34.00	9.00	15.00	69.00	84.00	596.00	49.00	20.00
Hisar Mukta	51.00	0.96	6.00	10.00	12.00	35.00	8.00	19.00	65.00	101.00	751.00	48.00	16.00
CO-2	49.00	0.98	5.00	8.00	12.00	21.00	10.00	17.00	69.00	74.00	784.00	48.00	17.00
AM-1	50.00	1.03	5.00	10.00	11.00	30.00	10.00	17.00	68.00	102.00	704.00	66.00	22.00
Hisar Madhavi	49.00	0.98	5.00	10.00	12.00	49.00	11.00	18.00	66.00	94.00	516.00	42.00	13.00
GM-2	50.00	1.00	5.00	9.00	11.00	30.00	9.00	17.00	69.00	94.00	646.00	42.00	15.00
Rajendra Kranti	50.00	0.97	5.00	9.00	10.00	29.00	11.00	17.00	62.00	93.00	516.00	45.00	16.00
RMT-143	50.00	0.92	6.00	12.00	10.00	36.00	11.00	18.00	68.00	86.00	538.00	44.00	14.00
Lam Selection	50.00	0.97	5.00	10.00	11.00	37.00	10.00	17.00	67.00	102.00	696.00	43.00	15.00
RMT-1	49.00	1.06	6.00	11.00	10.00	31.00	10.00	18.00	67.00	102.00	728.00	45.00	16.00
RMT-305	50.00	1.24	5.00	10.00	11.00	33.00	8.00	16.00	67.00	72.00	430.00	30.00	16.00
RMT-303	50.00	0.97	5.00	9.00	11.00	32.00	12.00	17.00	69.00	80.00	704.00	46.00	15.00
Pant Ragini	73.00	1.39	5.00	10.00	11.00	44.00	11.00	17.00	66.00	102.00	430.00	42.00	12.00
Mean	51.17	1.00	5.29	9.52	11.29	33.47	10.05	17.29	66.88	92.88	618.53	46.18	15.76
Standard Deviation	5.67	0.14	0.47	1.28	0.84	6.41	1.09	1.10	2.06	10.71	109.30	8.62	2.39
CV	11.08	14.36	8.87	13.45	7.44	19.16	10.83	6.36	3.08	11.53	17.67	18.66	15.13
S E M	1.37	0.03	0.11	0.31	0.21	1.56	0.26	0.26	0.50	2.60	26.51	2.09	0.58

1 Days to 50 % Flowering, 2 Petiole Length (cm), 3 No. of Primary Branches, 4 No. of Secondary Branches, 5 Pod Length (cm), 6 No of Pods/ Plant, 7 No. of Pods/axis , 8 No of Seeds/Pod, 9 Shelling %, 10 Days to 75% maturity, 11 Yield/plant (g), 12 Plant Height (cm), 13 Test Weight (g).

Genetic similarity matrix was calculated on the basis of Jaccards algorithm for RAPD data. The pair wise similarity values ranged between 0.43and 0.89 (Table 4). The maximum similarity value was noticed between RMT-303 and RMT-305 and minimum similarity values were between Azad Methi and RMT-1. The wide range of similarity indicated the presence of high genetic diversity in Indian fenugreek varieties.

The cluster analysis was conducted by the software NTSYS-pc version 2.20f (Rohlf, 1998). Similarity matrices based on Jaccard's similarity coefficient was used to construct the unweighted pair group method with arithmetic average (UPGMA) dendrogram (Fig 2). Among 17 varieties of fenugreek the similarity value ranges from 0.55 to 0.89 which exhibits a large amount of genetic diversity among them. A perusal of dendrogram indicates that there were two clusters; Cluster I consists of twelve genotypes which is again subdivided into five group A, B, C, D and E. Group IA consist of one variety Hisar Suvarna, group IB consists two varieties viz., Hisar Sonali and Rajendra Kranti, group IC consists five varieties viz., AM-2, Hisar Mukta, Hisar Madhavi, GM-2, AM-1 and Azad Methi, group ID consists two varieties namely CO-2 and RMT-143 where as group IE consists only one variety RMT-351. Cluster II consists of five varieties viz., Lam Selection, RMT-1, RMT-305, RMT-303 and Pant Ragini. RAPD analysis on fenugreek indicated the maximum similarity between RMT-303 and RMT-305 varieties which was shown by the coefficient of 0.89%. The high level of polymorphism observed is in conformity with the results of previous studies carried out on Indian fenugreek (Kakani *et al.*, 2011 and Dangi *et al.*, 2004). Number of bands obtained per primer indicated the efficiency of the primers to characterize the germplasm. The polymorphic

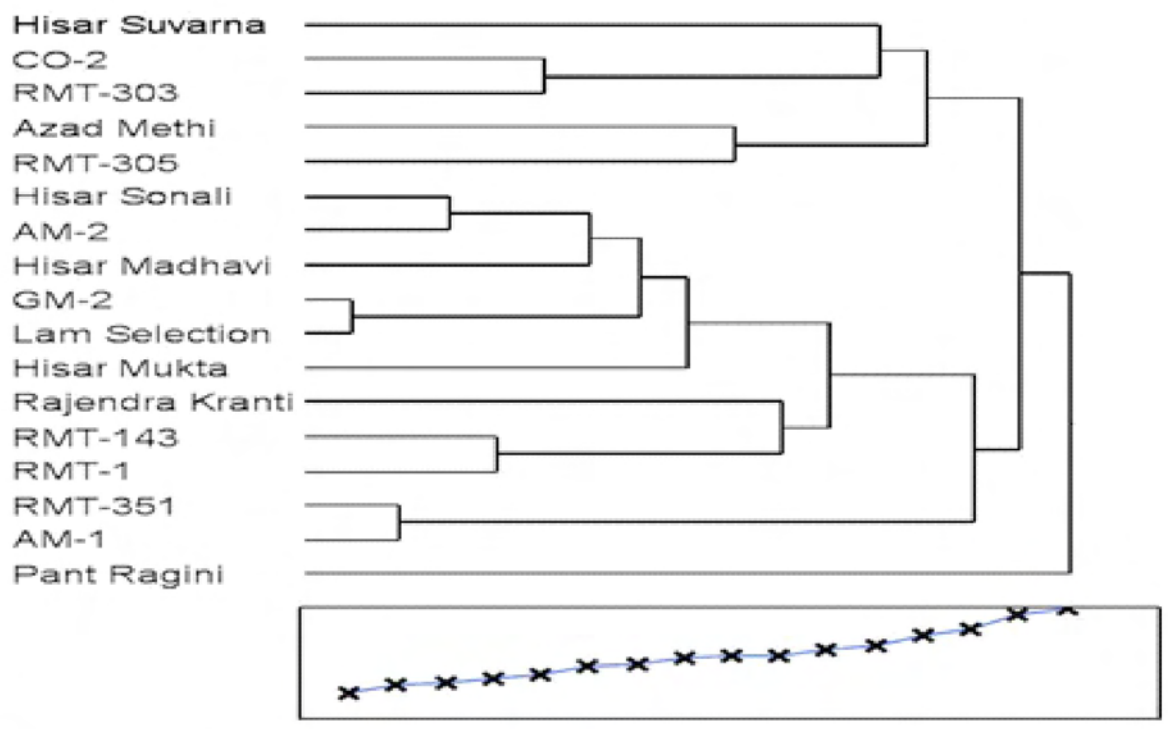

Fig. 1: Dendrogram based on Morphological character of seventeen varieties on fenugrek.

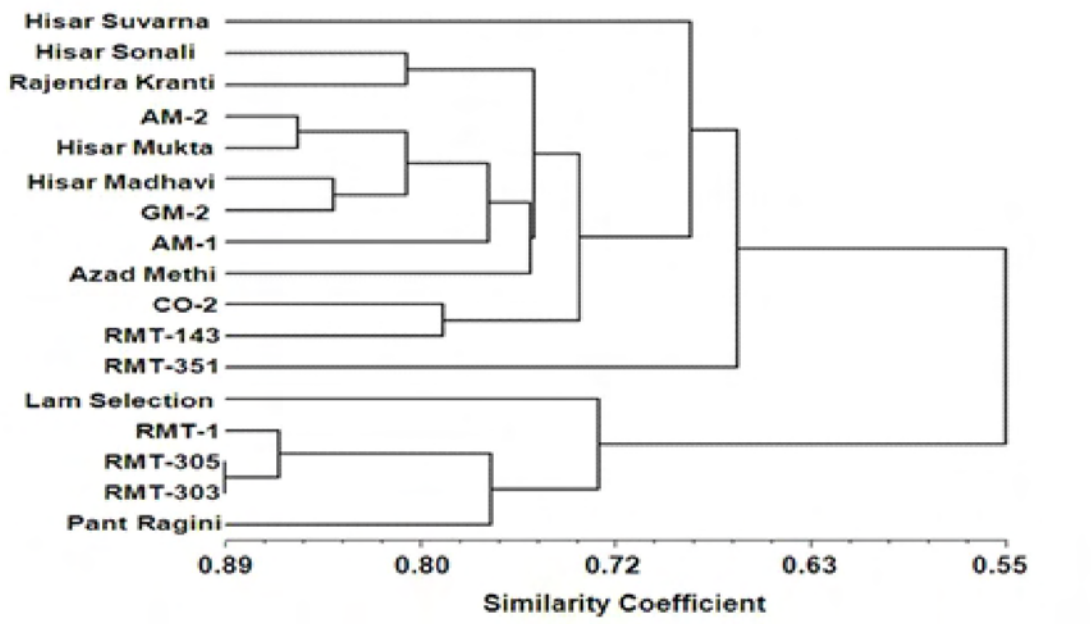

FIG. 2: Dendrogram illustrating the genetic relationships among 17 fenugreek varieties derived from an UPGMA cluster analysis based on Jaccard's similarly coefficient matrix, based on RAPD analysis. Scale at the bottom depicts the similarity values obtained using Jaccard's similarity coefficient.

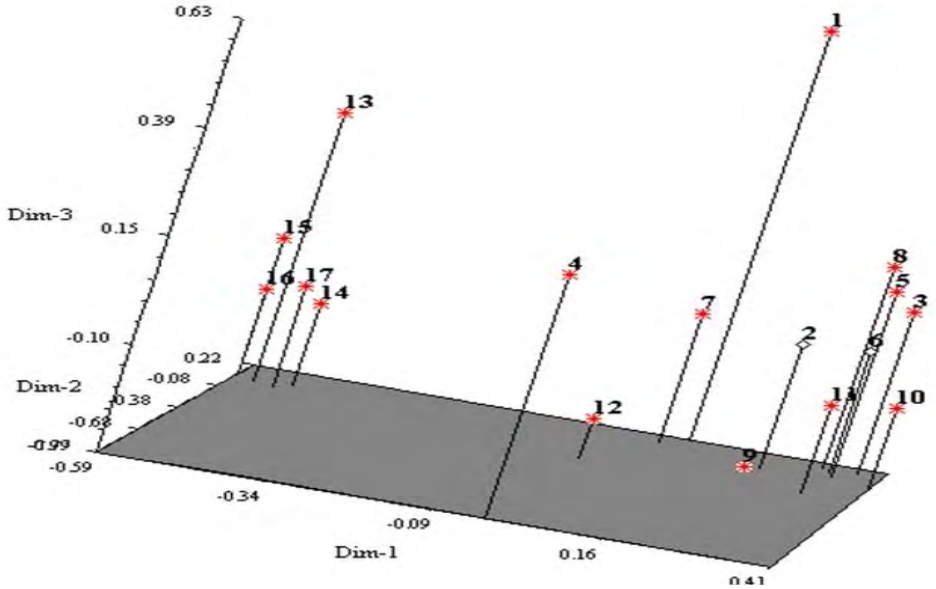

FIG. 3: Three dimentional graph based on PCA using RAPD markers. 1 Hisar Suvarna, 2 Hisar Sonali, 3 AM-2, 4 RMT-351, 5 Azad Methi, 6 Hisar Mukta, 7 Co-2, 8 Am-1, 9 Hisar Madavi,10 GM-2, 11 Rajendra Karnti, 12 RMT-143, 13 am Selection, 14RMT-1, 15 RMT-305,16 RMT-303, 17 Pant Ragini

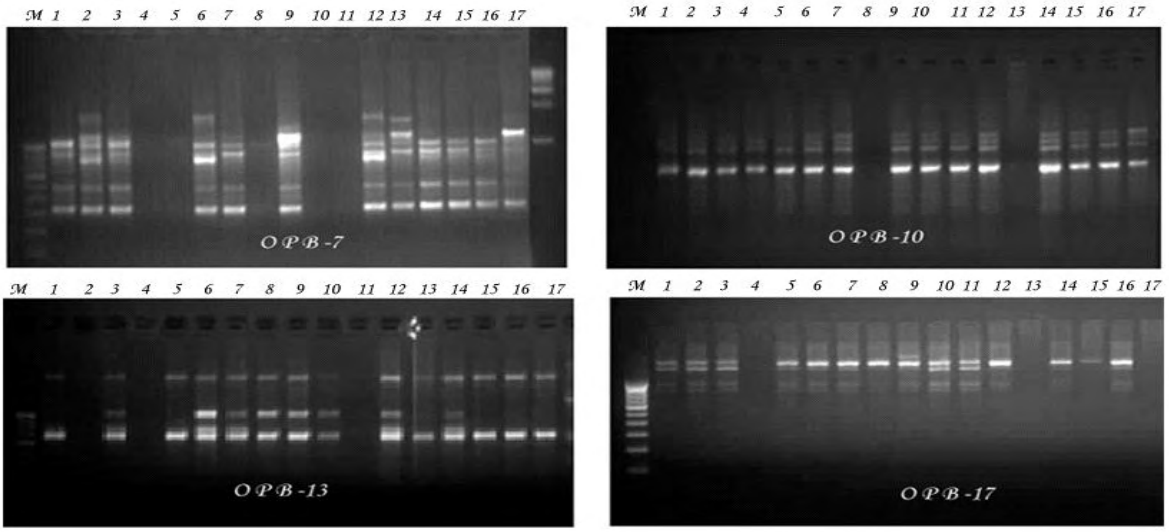

FIG. 4: RAPD profile of seventeen varieties of Fenugreek obtained with primer OPB-7, OPB 10, OPB-13 and OPB-17.

and the extent of information generated from RAPD analysis varied with the primers. The observation of molecular data reveals significant levels of diversity among genotypes. The greatest advantage of the RAPD approach is its technical simplicity and the independence of any prior DNA sequence information. The polymorphism indicated the effectiveness of at the intra specific level. The data generated by RAPD analysis was also supported by the PCA analysis. Principal Co-ordinate Analysis was performed to check the similarities and dissimilarities of the fenugreek population using the RAPD markers. Here main three clusters have been identified. In one cluster Lam Selection, Rajendra

TABLE 3: Clustering history for the morphological data of fenugreek population.

Number of Clusters	Distance	Leader	Joiner
16	1.500	GM-2	Lam Selection
15	1.889	RMT-351	AM-1
14	2.021	Hisar Sonali	AM-2
13	2.313	RMT-143	RMT-1
12	2.455	CO-2	RMT-303
11	2.936	Hisar Sonali	Hisar Madhavi
10	3.080	Hisar Sonali	GM-2
9	3.379	Hisar Sonali	Hisar Mukta
8	3.476	Azad Methi	RMT-305
7	3.501	Rajendra Kranti	RMT-143
6	3.807	Hisar Sonali	Rajendra Kranti
5	4.091	Hisar Suvarna	CO-2
4	4.699	Hisar Suvarna	Azad Methi
3	5.004	Hisar Sonali	RMT-351
2	5.738	Hisar Suvarna	Hisar Sonali
1	6.130	Hisar Suvarna	Pant Ragini

TABLE 4: Genetic similarity matrix for fenugreek varieties generaed using Jaccard's similarity coefficient.

	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q
A	1.00																
B	0.70	1.00															
C	0.76	0.78	1.00														
D	0.62	0.64	0.57	1.00													
E	0.67	0.71	0.75	0.64	1.00												
F	0.68	0.76	0.86	0.59	0.79	1.00											
G	0.76	0.75	0.75	0.67	0.75	0.79	1.00										
H	0.70	0.68	0.78	0.67	0.75	0.73	0.71	1.00									
I	0.62	0.76	0.79	0.68	0.70	0.78	0.79	0.76	1.00								
J	0.68	0.76	0.86	0.71	0.79	0.81	0.76	0.83	0.84	1.00							
K	0.64	0.81	0.75	0.76	0.78	0.73	0.75	0.75	0.76	0.83	1.00						
L	0.65	0.70	0.73	0.78	0.64	0.75	0.79	0.64	0.78	0.75	0.73	1.00					
M	0.60	0.49	0.46	0.64	0.46	0.48	0.62	0.52	0.54	0.48	0.52	0.67	1.00				
N	0.54	0.59	0.52	0.57	0.43	0.51	0.65	0.56	0.64	0.57	0.52	0.64	0.68	1.00			
O	0.60	0.59	0.49	0.57	0.46	0.48	0.68	0.52	0.57	0.51	0.52	0.64	0.75	0.87	1.00		
P	0.52	0.54	0.48	0.56	0.51	0.52	0.70	0.51	0.62	0.52	0.54	0.65	0.76	0.86	0.89	1.00	
Q	0.51	0.49	0.46	0.57	0.49	0.51	0.65	0.46	0.57	0.51	0.49	0.67	0.71	0.71	0.78	0.83	1.00

A Hisar Suvarna, **B** Hisar Sonali, **C** AM-2, **D** RMT-351, **E** Azad Methi, **F** Hisar Mukta, **G** Co-2, **H** Am-1, **I** Hisar Madhavi, GM-2, **K** Rajendra Karnti, **L** RMT-143, **M** Lam Selection, **N** RMT-1, **O** RMT-305, **P** RMT-303, **Q** Pant Ragini

Kranti, RMT-143, RMT-303, RMT-305 falls, in another cluster Hisar Suvarna, Hisar Sonali, AM-2, Azad Methi, Hisar Mukta, CO-2, Hisar Madhavi, GM-2 falls and in third RMT-351 and RMT-143 falls. PCA result clearly supports the finding of RAPD analysis (dendrogram). In PCA the group second and third has also no too significant differences as compared to RAPD dendrogram. The varieties with diverse pattern for RAPD and PCA are suggested to be used for further study and to select parents for inheritance or linkage groups (Eujayl *et al.*, 1998).

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